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L5 ANSWER 12 OF 47 MEDLINE DUPLICATE 7
AU Archer T K; Fryer C J; Lee H L; Zaniewski E; Liang T; Mymryk J S
TI Steroid hormone receptor status defines the ***MMTV*** ***promoter*** chromatin structure in vivo.
SO JOURNAL OF STEROID BIOCHEMISTRY AND MOLECULAR BIOLOGY, (1995 Jun) 53 (1-6) 421-9. Ref: 54
Journal code: AX4. ISSN: 0960-0760.

L5 ANSWER 13 OF 47 MEDLINE DUPLICATE 8
AU Xu A; Kudo S; Fukuda M
TI A novel expression vector composed of a regulatory element of the human leukosialin-encoding gene in different types of mammalian cells.
SO GENE, (1995 Jul 28) 160 (2) 283-6.
Journal code: FOP. ISSN: 0378-1119.

L5 ANSWER 14 OF 47 MEDLINE DUPLICATE 9
AU Petitclerc D; Attal J; Theron M C; Bearzotti M; Bolifraud P; Kann G; Stinnakre M G; Pointu H; Puissant C; Houdebine L M
TI The effect of various introns and transcription terminators on the efficiency of expression vectors in various cultured cell lines and in the mammary gland of transgenic mice.
SO JOURNAL OF BIOTECHNOLOGY, (1995 Jun 21) 40 (3) 169-78.
Journal code: AL6. ISSN: 0168-1656.

L5 ANSWER 15 OF 47 MEDLINE DUPLICATE 10
AU Wilson S E; Weng J; Blair S; He Y G; Lloyd S
TI Expression of E6/E7 or SV40 large T antigen-coding oncogenes in human corneal endothelial cells indicates regulated high-proliferative capacity.
SO INVESTIGATIVE OPHTHALMOLOGY AND VISUAL SCIENCE, (1995 Jan) 36 (1) 32-40.
Journal code: GWI. ISSN: 0146-0404.

L5 ANSWER 17 OF 47 CAPLUS COPYRIGHT 2000 ACS DUPLICATE 12
AU Pendse, Girish J.; Bailey, James E.
TI Effect of Vitreoscilla hemoglobin expression on growth and specific tissue plasminogen activator productivity in recombinant Chinese hamster ovary cells
SO Biotechnol. Bioeng. (1994), 44(11), 1367-70
CODEN: BIBIAU; ISSN: 0006-3592

L5 ANSWER 18 OF 47 MEDLINE DUPLICATE 13
AU Archer T K; Zaniewski E; Moyer M L; Nordeen S K
TI The differential capacity of glucocorticoids and progestins to alter chromatin structure and induce gene ***expression*** in ***human*** breast cancer ***cells*** .
SO MOLECULAR ENDOCRINOLOGY, (1994 Sep) 8 (9) 1154-62.
Journal code: NGZ. ISSN: 0888-8809.

Communication to the Editor

Effect of Vitreoscilla *Hemoglobin Expression on Growth and Specific Tissue Plasminogen Activator Productivity in Recombinant Chinese Hamster Ovary Cells*

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Previous studies suggest that secretion of cloned proteins synthesized by recombinant Chinese hamster ovary (CHO) cells can be adenosine triphosphate (ATP) limited. Other research indicates that the presence of cloned *Vitreoscilla* hemoglobin (VHb) enhances ATP production in oxygen-limited *Escherichia coli.* To evaluate the influence of VHb expression on recombinant CHO cell productivity, the *vhb* gene has been fused to the mouse mammary tumor virus (MMTV) promoter and cloned in a CHO cell line previously engineered to express human tissue plasminogen activator (tPA). Western blot analysis confirms dexamethasone-inducible VHb expression in all of the clones tested. Batch cultivation experiments with one VHb-expressing clone and the parental CHO–tPA cells show a reduced specific growth rate in the VHb-expressing cells. The VHb-expressing clone exhibits specific tPA production 40 to 100% greater than the parental CHO–tPA culture.
Key words: CHO cells • cloned proteins • VHb hemoglobin

INTRODUCTION

Under oxygen-limited conditions, expression of *Vitreoscilla* hemoglobin (VHb) in *Escherichia coli* resulted in faster growth, considerably higher cell densities, increased respiration rates and increased protein synthesis in fed-batch fermentations.[4,5,7] These data suggest that intracellular expression of VHb improves the overall efficiency of oxygen-limited adenosine triphosphate (ATP) production in oxygen-limited *E. coli,*[8] a phenomenon subsequently demonstrated directly using in vivo nuclear magnetic resonance (NMR) spectroscopy.[3] Many studies with a wide range of mammalian hosts as well as different heterologous proteins have shown that high-level expression can be limited by bottlenecks in the post-translational secretory pathway. In particular, in vivo dissociation from GRP78 may be an ATP-dependent, rate-limiting step in transport of cloned proteins from the endoplasmic reticulum.[1,2,11] The possibility that expression of *Vitreoscilla* hemoglobin in recombinant Chinese hamster ovary (CHO) cells might accelerate a limiting step in post-translational processing of product was the motivation for this research.

MATERIALS AND METHODS

Bacterial Strains and Plasmid Vectors

Escherichia coli strain HB101 was used for all routine bacterial culture work including minipreps and maxipreps for isolation of plasmid DNA preparations. The vector pMSG was obtained from Pharmacia Biotechnology (Piscataway, NJ). The source of the *Vitreoscilla* hemoglobin structural gene was pRED302 (Chaitan Khosla, personal communication), a derivative of pRED2.[5] All restriction enzymes used in this study were obtained from Boehringer Mannheim (Indianapolis, IN).

Construction of pMSG-VHb

The *vhb* structural gene was obtained by digesting pRED302 with *Xba*I and *Ssp*I. A large fragment of pMSG was isolated and purified by sequentially digesting pMSG with *Nhe*I and *Sma*I. An overnight blunt-sticky end ligation was carried out at 8 to 15°C for cloning the *XbaI/SspI vhb* fragment into the *NheI/Sma*I-digested pMSG to obtain pMSG–VHb. Appropriate digestion reactions and subsequent agarose gel electrophoresis experiments using DNA from HB101 transformants confirmed the authenticity of the new construct. Large amounts of pMSG–VHb DNA required for CHO cell transfection were obtained by standard maxiprep protocols.

C ll Culture and CHO Cell Transfecti n

Chinese hamster ovary cells producing tissue plasminogen activator (tPA) (ATCC 9606) were obtained from the Amer-

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ican Type Culture collection (ATCC) (Bethesda, MD). These cells were passaged routinely in nonselection medium containing Dulbecco's modified Eagle's medium (DMEM) (high glucose) (GIBCO, Grand Island, NY) supplemented with 1× penicillin–streptomycin–glutamine (Irvine Scientific) solution and 5% dialyzed fetal bovine serum (FBS) (GIBCO, Grand Island, NY) in a 5% CO_2 humidified incubator at 37°C. Tissue culture dishes (100 × 20 mm) were used in all experiments. The selection medium for transfected cells contained 25 mg/L mycophenolic acid (GIBCO, Grand Island, NY), 1× hypoxanthine aminopterin thymine (HAT); (GIBCO, Grand Island, NY), and 250 mg/L xanthine (Sigma, St. Louis, MO) in addition to the nonselection medium components. During regular cultures, cells were passaged every 2 to 3 days upon reaching semiconfluency. Total cell count and cell size distribution were monitored by a Coulter counter. Cell viability was determined using the trypan blue exclusion method. Transfection of parental CHO cells was done using the calcium phosphate procedure described by Maniatis et al.[10]

About 40 colonies resistant to the mycophenolic acid–xanthine selection pressure were picked using cotton tips wetted with trypsin and transferred to 24-well tissue culture plates. After expanding these to 60-mm dishes, all colonies except three were frozen down at −70°C. Three clones (clones 4, 19, and 30) were gradually expanded to 100-mm dishes for further studies. The presence of the *vhb* gene in the CHO cell chromosomes and inducible expression of VHb protein in these transfected clones was confirmed using Southern hybridization and Western blots, respectively, which are discussed later. After these initial confirmatory tests, only the parental CHO–tPA and one VHb expressing clone were used for detailed physiological characterization studies.

Dose–Response Experiments

Cells were inoculated at 4×10^5 cells/dish. Twenty-four hours postinoculation, dexamethasone (Sigma, St. Louis, MO) was added to each dish at final concentrations ranging from 0 to 1 μM. Cell growth was monitored every day as described earlier. Cell extracts were prepared for Western blot analysis as described in the following section. In one experiment, the VHb expression was monitored as a function of dexamethasone concentration (0, 0.1, 0.2, 0.5, and 1.0 μM) after 48 h of induction. In another experiment, VHb expression was monitored for a constant dexamethasone concentration (0.1 and 0.5 μM) for induction times of 24, 48, 72, and 96 h.

SDS-PAGE and Western Blot Analysis

Cell extracts were prepared for each sample following the protocol provided by Exogene Inc. (Monrovia, CA). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using standard protocols using the Protean mini-gel apparatus (Bio-Rad, Richmond, CA). The proteins were transferred to a nitrocellulose membrane and VHb detection was performed using antiserum against VHb.[6] *Escherichia coli* JM101:pRED2[5] (this strain expresses high levels of VHb) cell extract was used as VHb standard.

Southern Hybridizati n

Genomic DNA was isolated from various clones using standard protocols from Maniatis.[10] Five micrograms of DNA each of parental CHO–tPA and the three VHb–CHO clones was digested overnight with *EcoR*I at 37°C, electrophoresced and transferred overnight to an Immobilon-S membrane (Millipore, Bedford, MA) using the capillary blot technique.[10] The VHb gene probe isolated by minipreps was labeled with biotin using a PolarPlex Kit (Millipore, Bedford, MA). Prehybridization and hybridization were carried out overnight in a 68°C waterbath according to the PolarPlex protocol. The following day, detection reaction was performed using the Lumigen reagent. A permanent image of the hybridization pattern was obtained by exposing the membrane to an X-ray film for time periods ranging from 10 min to 1 h.

tPA Assay

Tissue plasminogen activator production during culture was monitored using an enzyme-linked immunosorbent assay (ELISA) kit (COELIZA, KabiVitrum, Franklin, OH) according to the standard protocol provided by the manufacturer. The total amount of tPA produced each day was calculated by multiplying the concentration of tPA obtained by ELISA by the volume of supernatant present in each dish. This was done to account for the progressive reduction in volume of the supernatant due to evaporation of water during the course of the batch culture.

RESULTS

Dose–Response Experiments

A tPA-expressing CHO cell line (ATCC 9606) was transfected with the vector pMSG–VHb which should provide dexamethasone-inducible VHb expression.[9] Induction experiments were done with the parental CHO–tPA cell line as well as the three VHb–CHO clones (designated as 30, 4, and 19). Results of Western blots from dose–response experiments for the VHb–CHO clone 30 are shown in Figure 1. Although the uninduced sample (no dexamethasone) shows a faint band corresponding to VHb, we observed significant induction of the MMTV promoter for dexamethasone concentration of 0.1, 0.2, 0.5, and 1.0 μM. Dexamethasone, 0.1 μM, is sufficient for full induction of VHb expression. VHb expression was observed at 24, 48, 72, and 96 h postinduction (Fig. 2). The amount of sample loaded in each lane was not the same, so no conclusions are

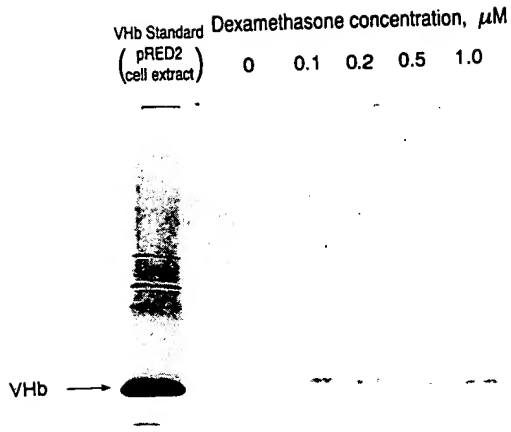

Figure 1. Western blot for detection of VHb expression in clone 30 as a function of dexamethasone concentration. Dexamethasone concentrations ranging from 0 to 1 m*M* were used in this experiment. Cell extracts were prepared at 48 h postinduction.

possible from the intensity of the VHb bands in different lanes. No protein hybridizing to VHb antiserum was observed for induced or uninduced parental CHO cells. Similar patterns of VHb expression were observed in the VHb–CHO clones 4 and 19 (data not shown).

Southern Hybridization

All VHb–CHO clones show two distinct hybridization bands whereas the parental clone shows one band when probed with the *vhb* gene. The lower band is present in all four clones, suggesting that this is a fragment of the genomic DNA with substantial homology with the *vhb* gene. The upper band is present only in the VHb–CHO clones which presumably corresponds to the *vhb* gene. The upper band is present at a different location in clones 4 and 19 as compared to clone 30. However, both of these clones express VHb in a manner similar to clone 30 (data not shown).

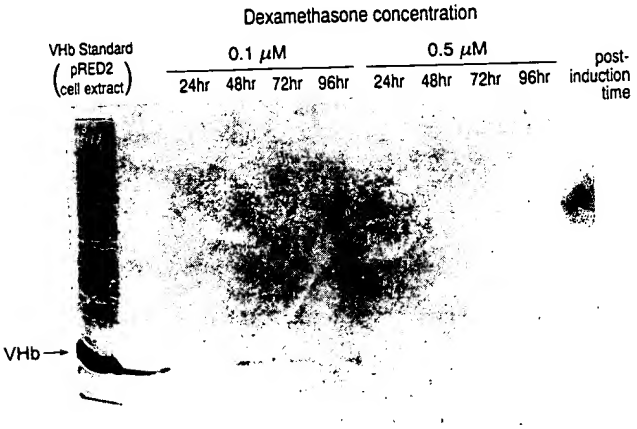

Figure 2. Western blot for detection of VHb expression in clone 30 as a function of time postinduction. Dexamethasone concentrations of 0.1 and 0.5 μ*M* were used in this experiment. Cell extracts were prepared at 24, 48, 72, and 96 h postinduction.

Batch Culture Experiments

Once the cloning of the *vhb* gene and its expression in CHO cells to give intracellular VHb protein was established, we studied the effect of VHb expression on cell growth and tPA productivity in batch cultures. Each batch culture experiment was carried out twice independently. Figure 3A and B show the growth curves obtained during the two independent runs with control and VHb-expressing clones under different induction conditions. Figure 4 shows the specific tPA production averaged for the two runs, expressed as total tPA produced/10^6 cells.

The specific growth rate of VHb–CHO cells is about 20 to 30% lower than that of the parental CHO cells. However, this effect does not seem to be due to VHb expression since the uninduced VHb–CHO cells have almost the same growth characteristics as the induced VHb–CHO cells. Furthermore, the growth curves were almost the same for both induced and uninduced parental CHO cells up to a dexamethasone concentration of 1 μ*M*. Thus, in this range of conditions, dexamethasone does not appear to have any significant effect on observed cell physiology.

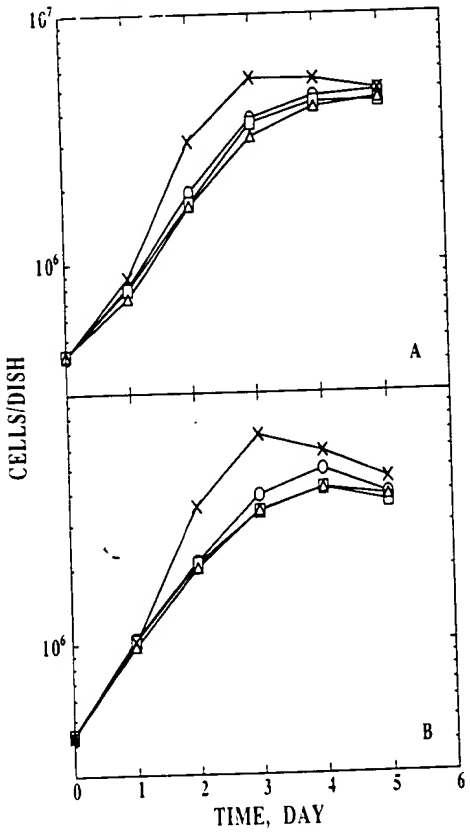

Figure 3. Total cell number/dish in batch culture from (A) run 1 and (B) run 2. Cells were inoculated at 4 × 10^5 cells/100 mm tissue culture dish: (×) CHO–tPA, (○) VHb–CHO/0 μ*M* dexamethasone, (□) VHb–CHO/0.1 μ*M* dexamethasone, (△) VHb–CHO/0.5 μ*M* dexamethasone.

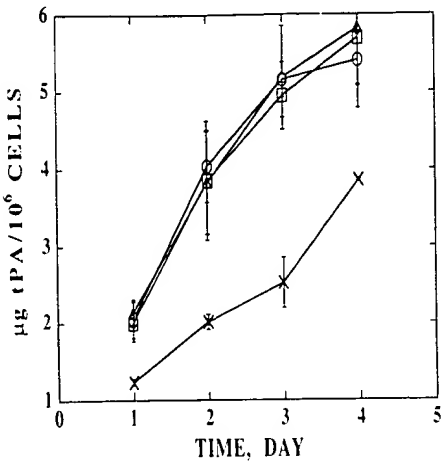

Figure 4. Specific tPA production in batch culture averaged for runs 1 and 2: (×) CHO–tPA, (○) VHb–CHO/0 µ*M* dexamethasone, (□) VHb–CHO/0.1 µ*M* dexamethasone, (△) VHb–CHO/0.5 µ*M* dexamethasone.

The tPA productivity characteristics are significantly different in the VHb–CHO clone compared to the parental CHO–tPA cells. The total amount of tPA produced by VHb–CHO cells was either equal to or higher than that produced by CHO–tPA cells throughout the batch (data not shown). The specific tPA production is 40 to 100% higher in VHb–CHO cells compared to the parental CHO–tPA cells.

DISCUSSION AND CONCLUSION

The productivity of recombinant CHO cells has been increased by cloning into these cells the gene coding *Vitreoscilla* hemoglobin in an inducible mammalian expression vector. However, we cannot completely exclude the possibility that this enhanced production might be due to some other host–vector interaction. This productivity enhancement is observed even for uninduced VHb–CHO cells. However, we have shown the MMTV promoter to be leaky so that some basal VHb expression is obtained even in the uninduced cells. It is possible that the uninduced level of intracellular VHb is enough to cause the beneficial effects that we observe.

Unfortunately, VHb dose–physiological response experiments have not yet been reported for any of the systems in which VHb has so far been expressed. Two-dimensional analytical protein gel electrophoresis assays of samples taken from fed-batch cultivations of VHb-expressing *E. coli* indicate major physiological effects of VHb presence at expression levels of less than 1% of total cell protein.[8] Responses to lower VHb expression levels have not yet been tested. These data indicate that, in *E. coli*, VHb does not serve any bulk oxygen storage function but instead contributes in some catalytic role to improved growth under oxygen-limited conditions.

No lower limit of VHb expression below which the metabolic effects of VHb diminish has been detected or defined to date in any strain or cell line. Therefore, the uninduced VHb expression level observed with VHb–CHO clones could well be sufficient to obtain a metabolic response which is already saturated with respect to the intracellular level of VHb. Although the increased specific productivity observed here cannot be rigorously attributed solely to the presence of VHb, given the metabolic effects of VHb in other systems, its demonstrated role in enhancing ATP production in *E. coli*,[3] and possible ATP limitations in post-translational processing of cloned proteins in eukaryotic cells, these positive results encourage studies of the possible benefits of VHb in other eukaryotic expression systems.

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